Pseudorabies: Adaptation of the Countercurrent Immunoelectrophoresis for the Detection of Antibodies in Porcine Serum

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ABSTRACT

A countercurrent immunoelectrophoresis test was developed for the detection of precipitating antibodies to pseudorabies virus in pig serum. The precipitation reaction occurred only between the pseudorabies antigen and the homologous porcine antiserum. The sensitivity of the method was compared to that of the serum neutralization test. On the basis of its sensitivity, its specificity and the rapidity with which the results were obtained, the countercurrent immunoelectrophoresis may become a potentially valuable screening method to test large numbers of porcine serum.

RÉSUMÉ

Les auteurs ont mis au point l'épreuve de l'immunosynérèse dans le but de détecter les anticorps précipitants dans le sérum de porcs infectés avec le virus de la pseudorage. La réaction de précipitation s'est avérée spécifique puisqu'elle est apparue uniquement entre l'antigène de la pseudorage et le sérum immun correspondant. La sensibilité de la méthode était comparable à celle de l'épreuve de séro-neutralisation. Tenant compte de sa sensibilité, de sa spécificité et de la rapidité avec laquelle les résultats ont été obtenus, l'immunosynérèse est proposée comme une méthode de tamisage intéressante, lorsqu'il faut procéder à l'épreuve d'un grand nombre d'échantillons de sérum de porcs.

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INTRODUCTION

The value of the serum neutralization (SN) test in the serodiagnosis of pseudorabies infection in pigs has long been recognized (8). However, the frequent occurrence of cytotoxicity and the presence of contamination and hemolysis in some porcine serums hamper the diagnosis of pseudorabies by the SN test. A double immunodiffusion test (DIDT), which is less affected by these factors, was proposed as an alternative to the SN test. The sensitivity of the DIDT was reported to be similar to that of the SN test. Furthermore, in positive samples, the precipitation reactions occurred sooner (12-34 hr) than the development of cytopathic effect (CPE) in cell cultures (48-72 hr) (7).

A test that would combine the advantages of DIDT and could produce the results faster would be an asset to a diagnostic laboratory. Such advantages are offered by the countercurrent immunoelectrophoresis (CIE) test, in which both the antigen and the antibody are electrophoresed simultaneously (2). The CIE test has been adapted for routine serodiagnosis of aleutian disease in mink (3) and human viral hepatitis (10) but to our knowledge it has not yet been reported for pseudorabies.

The purpose of this investigation was to adapt the CIE test to detect antibodies to pseudorabies virus in pig serums and to assess its reliability in comparison with the SN test.

MATERIALS AND METHODS

VIRUS

The pseudorabies virus strain, purchased

from the American Type Culture Collection[1], was passaged once in monolayer cultures of primary pig kidney (PK) cells and stored at —70°C until used. This first passage was used in SN tests in the preparation of antigen and to infect pigs.

Pigs

Pigs were obtained from the swine herd of the Animal Diseases Research Institute (E). This herd, established from SPF animals and maintained in isolation, is monitored regularly for the presence of extraneous agents (4). It is referred to as the minimal disease (MD) swine herd.

Cell Cultures

Monolayer cultures of primary PK cells were prepared according to the method outlined by Greig *et al* (6). The IB-RS-2 porcine kidney cell line was obtained from D.W.G. Chapman[2]. Both cell cultures were grown in 0.5% lactalbumin hydrolysate supplemented with 0.01% yeast extract and 10% fetal bovine serum (FBS).

Pseudorabies Virus Antigen

Monolayer cultures of PK cells grown in disposable Brockway bottles[3] were infected with pseudorabies virus suspension. The inoculum was allowed to adsorb onto the monolayers for one hr at 37°C then maintenance medium (MEM in Earle's salts without FBS) was added and the flasks were returned to the incubator until the cells showed extensive CPE (36 to 48 hr). The virus was released from the cells by three cycles of freezing and thawing. The fluids were clarified by centrifugation in the cold at 1000 X g for 30 min. The supernatant fluids from infected cell cultures were treated with ammonium sulfate as described by Gutekunst *et al* (7). In some cases, however, the supernatant fluids were inactivated by acetyl ethylene imine (AEI) prior to precipitation with ammonium sulfate (9). Precipitates from both the infectious and the inactivated fluids were sedimented by low speed centrifugation and the supernatant fluids were discarded. The pellet was dissolved in distilled water (1/100 of the original volume) and dialyzed against several changes of distilled water. The dialysate was concentrated approximately 150 times the original volume with polyethylene glycol MW 20,000 and centrifuged at 20,000 X g for one hr with an IEC centrifuge in the A-321 rotor[4]. The clear supernatant fluid was dialyzed against veronal buffer, pH 8.2 (same dilution in the electrophoretic gel, see in countercurrent immunoelectrophoresis) and was used without further purification in the CIE test. This preparation was referred to as the positive antigen. Noninoculated PK cells were processed similarly and used as negative antigen. The antigens were tested for infectivity by the method of Anderson *et al* (1).

Antiserum

Two groups of pigs, weighing approximately 30 kg each, were infected with pseudorabies virus. The three pigs, assigned to the first group, received 1000 $TCID_{50}$ of virus suspension (1 ml) administered intranasally. Two days later the pigs of this first group were transferred to another stall with two noninoculated contact pigs. Both groups of pigs were then kept together until the end of the experiment. Each animal was bled prior to inoculation and test bled subsequently according to the schedule presented in Table I. Basically, the animals were bled weekly for the first two months and monthly for the subsequent five months then bimonthly until the end of the experiment.

Each blood sample was analyzed by the SN and by CIE tests for comparison. Following preliminary tests, serum P-34, 302 dpi was found to produce the sharpest precipitation line with the positive antigen only. This serum was selected as reference

[1]American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

[2]The Animal Virus Research Institute, Pirbright, Woking, Surrey, GU24 DNF, England.

[3]Brockway Glass Company, Inc., 100 Brock Bldg., Brockway, Pennsylvania 15824.

[4]International Equipment Co., 300 Second Ave., Needham Heights, Massachusetts 02194.

TABLE I. Antibody Profile to Experimental Infection of Pigs with Pseudorabies Virus as Demonstrated by SN and CIE Tests

Days Post-inoculation	Group I Pigs Infected Intranasally P-34 SN	P-34 CIE	P-35 SN	P-35 CIE	P-36 SN	P-36 CIE	Days Post-exposure	Group II Contact Pigs P-1 SN	P-1 CIE	P-2 SN	P-2 CIE
0	—[a]	—	—	—	—	—	0	—	—	—	—
7	1:8[b]	+[c]	1:2	—	1:2	+	5	—	—	—	—
9	1:33	+	dead		1:4	+	7	—	—	—	—
12	1:52	+			1:12	+	10	dead		1:12	+
14	1:130	+			1:16	+	12			1:3	+
21	1:120	+			1:16	+	19			1:3	+
28	1:160	+			1:80	+	26			1:40	+
35	1:108	+			1:56	+	33			1:25	+
42	1:80	+			1:50	+	40			1:14	+
49	1:100	+			1:56	+	47			1:20	+
56	1:120	+			1:50	+	54			1:25	+
85	1:80	+			1:50	+	83			1:40	+
114	1:62	+			1:80	+	112			1:50	+
145	1:100	+			1:160	+	143			1:40	+
215	1:112	+			1:100	+	213			1:50	+
281	1:100	+			1:62	+	279			1:28	+
299	1:112	+			1:62	+	297			1:40	+

[a]Negative reaction
[b]50% neutralizing titers
[c]Positive reaction

to standardize the CIE system. Antiserum to porcine cytomegalovirus, with a CF titer of 1:80 was obtained from Dr. J. Stevens[5].

Field Serums

One hundred serums collected from Canadian swine and 50 field serums obtained from the United States and provided by Dr. Gutekunst[6] were assayed by SN and CIE tests.

Serum Neutralization Test

The SN test was done in flat bottom cell culture grade microtiter plates[7], using IB-RS-2 porcine cell-line as described by Golding *et al* (5). The test was performed according to the protocol prepared by the Pseudorabies Diagnostic Standardization Committee of the American Association of Veterinary Laboratory Diagnosticians, supplied by Dr. Howard Hill[6]. The 50% neutralizing endpoints of the serums were estimated according to the method of Reed and Muench (11).

Countercurrent Immunoelectrophoresis

Reagents were electrophoresed in a veronal buffer system, pH 8.2, ionic strength 0.05, in the electrophoretic chamber and a 1:2 dilution of the same buffer in the gel. The gel was a 1% agarose containing merthiolate at a dilution of 1:10,000. Ten ml of melted agarose was measured on each side of an immuno frame holding three glass slides of 25 x 75 mm[8]. After the gel was set, wells of 3 mm in diameter were cut and the plugs were removed by suction. The distance between the antigen and antibody wells was 4 mm (Fig. 1). Serums collected before and after infection were tested with the positive antigen and the negative antigen. The wells were filled with capillary tubes (10 μl in each well) and electrophoresis was carried out at 10 Volts/cm for 60 min in a Gelman Electrophoretic Chamber[9]. The undiluted and non-inactivated serum was positioned on the anodal and the antigen on the cathodal side. After electrophoresis, the slides were examined in indirect light for the presence of precipitation lines between antigen and serum wells.

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[6]National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 70, Ames, Iowa 50010.

[7]Micro Test II Tissue Culture plate No. 3040 Falcon, Div. Becton, Dickinson and Co., Oxnard, California 93030.

[8]Gelman Electrophoresis Kit (51446), Gelman Instrument Co., Ann Arbor, Michigan 48106.

[9]Gelman Instrument Co., Ann Arbour, Michigan 48106.

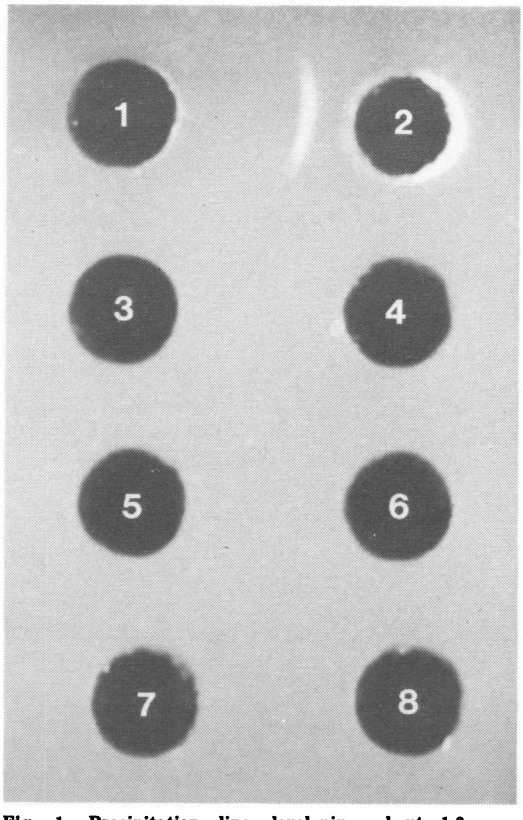

Fig. 1. Precipitation line developing about 1.2 mm from the antibody well. Positive antigen (1 and 5), negative antigen (3 and 7), positive serum (2 and 4), negative serum (6 and 8).

ANTIGEN TITRATION

A checkerboard titration of the positive antigen with the positive reference serum was carried out by the CIE test in order to establish the optimal concentration of antigen to be used in routine tests. One unit of antigen was defined as the highest dilution of antigen producing a visible line of precipitation with the highest dilution of serum. Four units of antigen were used in the test.

RESULTS

When infectious and inactivated pseudorabies viral antigens and homologous antiserums were electrophoresed for 30 min in a veronal buffer system, a precipitation line appeared only between wells of the positive antigen and positive serums. The line became stronger when the electrophoresis was continued for an additional 30 min. The precipitation line was about 1.2 mm from the antibody wells (Fig. 1). The positive antigen failed to react with preinoculation serums and similarly, no precipitation occurred between the negative antigen and positive serums. The results of the checkerboard titration of the pseudorabies antigen and the homologous antiserum are presented in Table III. The titer of the antigen was 1:64. The results of the SN and CIE tests obtained on sequential bleedings of pigs infected experimentally are summarized in Table I. Precipitating antibodies were detected at seven days postinoculation in two of the three (P-34 and P-36) pigs infected intranasally. The third pig (P-35) had an SN titer of 1:2 at seven days postinoculation but was negative by the CIE test. This animal died on day 8 without being bled. One of the contact pigs (P-1) died on day 8 but had no antibodies detectable by the CIE test on the seventh day. The other pig (P-2) had detectable levels of antibodies on day 10. All serum samples collected at later dates remained positive until the end of the experiment (299 days). None of the serums reacted with the negative antigen.

In comparison, neutralizing antibodies were detected at seven days in the three pigs infected intranasally, whereas detectable level of antibodies appeared on day 10 in the surviving contact pig (P-2). No precipitation line developed between antiserum to porcine cytomegalovirus and pseudorabies antigen. One hundred Canadian field serums found negative by the SN test were also negative with the CIE test and no nonspecific precipitation lines were encountered. Fifty field serums obtained from the U.S.A. were compared by both the SN and the CIE tests and the results are presented in Table II. It will be seen that all those serums that were found positive by the SN test were also positive by the CIE test except No. 47. And similarly, all those serums that were negative by the SN test failed to precipitate in the CIE test with the exception of No. 46.

DISCUSSION

The CIE test was adapted for the sero-

TABLE II. Demonstration of Antibody to Pseudorabies Virus in Field Serums by SN and CIE Tests

No. of Serum	SN Titer	CIE	No. of Serum	SN Titer	CIE
1	1:256	+[a]	26	1:32	+
2	1:64	+	27	1:8	+
3	1:16	+	28	1:64	+
4	1:16	+	29	1:32	+
5	1:16	+	30	1:32	+
6	1:32	+	31	1:64	+
7	1:16	+	32	1:2	+
8	1:128	+	33	1:16	+
9	1:16	+	34	1:16	+
10	1:16	+	35	1:256	+
11	1:64	+	36	1:16	+
12	1:16	+	37	1:16	+
13	1:32	+	38	1:16	+
14	1:32	+	39	1:4	+
15	1:16	+	40	1:16	+
16	1:4	+	41	1:16	+
17	1:8	+	42	1:4	+
18	1:8	+	43	1:8	+
19	1:64	+	44	−[b]	−
20	1:4	+	45	−	−
21	1:2	+	46	−	+
22	1:64	+	47	1:2	−
23	1:8	+	48	−	−
24	1:8	+	49	−	−
25	1:64	+	50	−	−

[a]Positive reaction
[b]Negative reaction

TABLE III. Checkerboard Titration of Pseudorabies Antigen and Antiserum by CIE Test

	Antigen Dilutions					
Antiserum Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
1:1	+[a]	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	+
1:8	−[b]	−	−	−	−	+
1:16	−	−	−	−	−	−

[a]Positive reaction
[b]Negative reaction

diagnosis of pseudorabies in pigs. The reaction was specific inasmuch as it appeared between the positive antigen and the homologous antiserum only. Cross reactions, detectable by double immunodiffusion, are known to occur between members of the herpes group of viruses (13). It is unlikely, however, that such cross reactions will interfere with the interpretation of results obtained by the CIE test. This view is supported by our findings in which there was no reaction between an antiserum to porcine cytomegalovirus and the positive pseudorabies antigen. Although two SN positive serums (one in the experimentally infected and the other in the group obtained from the U.S.A.) with a low (1:2) SN titer failed to react in the CIE test, the sensitivity of this method compared favourably with that of the SN assay, since all serums that had neutralizing titers of 1:3 or greater were also positive with the new method.

Circulating antibodies were detected by the CIE test as early as seven days post-infection and persisted throughout the experiment. These findings are in agreement with those of McFerran and Dow (8) and Skoda *et al* (12). These results suggest that the method is potentially valuable to screen large numbers of serums in a short time.

It can be mentioned also, not to the disadvantage of the CIE test, that all these factors that adversely influence the SN test, such as contamination or cytotoxicity,

do not affect the result of the CIE test. Hence, the CIE test is endowed with the good features of the DIDT and it also presents the added convenience of yielding results within one hour.

Since the pseudorabies antigen retained its reactivity in the CIE test following its inactivation with AEI large numbers of animals could be tested without endangering a susceptible animal population. This aspect is particularly important in countries where pseudorabies is considered to be exotic and the accidental escape of this agent would be detrimental to agriculture.

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ERRATUM

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A Study of the Role of Genetic Factors in the Etiology of Left Abomasal Displacement.

In Figure 1 (p. 513), the sires currently numbered 7, 6, 5 and 4, should be renumbered as sires 4, 5, 6 and 7 respectively.

The authors regret any inconvenience caused.